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Trehalose lowers membrane phase transitions in dry yeast cells

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Abstract

Recent work has clearly demonstrated a direct correlation between the amount of trehalose present in the yeast *Saccharomyces cerevisiae* and its ability to tolerate dehydration, but has failed to elucidate the specific role played by trehalose. By using Fourier transform infrared spectroscopy we measured the transition temperature of phospholipids in both intact *S. cerevisiae* and isolated plasma membranes dried in the presence and absence of trehalose. Our results show that trehalose lowers the temperature of the dry gel to liquid crystal phase transition in yeast from around 60°C to about 40°C, thus allowing yeast rehydrated above 40°C to avoid the damaging effects of passing through a phase transition. These results explain both the need for trehalose and the observation that yeast must be rehydrated with warm water if they are to remain viable. Only when trehalose is present is the dry transition within a physiologically tolerable range and only when the cells are rehydrated above 40°C will they avoid passing through a phase transition.

Key words: Trehalose; Phase transition; Plasma membrane; Dehydration; (*S. cerevisiae*)

1. Introduction

Trehalose is a non-reducing disaccharide (1-α-D-glucopyranosyl-1,1-α-D-glycopyranoside) found ubiquitously in fungi and widely in both bacteria and animals [1]. Trehalose is especially common in anhydrobiotic organisms, organisms capable of surviving extended periods of dehydration [2]. In the budding yeast *Saccharomyces cerevisiae* trehalose levels are low during periods of rapid growth and increase during periods of slow or no growth [3]. Although proposed to function as a stress protectant in anhydrobiotic organisms [4–6], trehalose was initially thought of as a reserve carbohydrate in yeast. Only recently has its role as a stress protectant in yeast been recognized [6,7], with several studies demonstrating a strong correlation between the amount of trehalose present and the viability of dried *S. cerevisiae* [8–10]. Although trehalose has been shown to be vital to yeasts' ability to survive desiccation, the mechanism of its action has remained unclear.

A possible explanation of the action of trehalose in dry yeast can be derived from model membrane studies. As phospholipid membranes are dried, the temperature at which the gel to liquid crystal phase transition (T_m) occurs increases [2]. Trehalose has been shown to interact with model membranes during drying and lower the dry membrane phase transition temperature [5,11]. Work on model systems has shown that dry liposomes rehydrated at room temperature, a temperature which is below the dry T_m, undergo a lyotropic phase transition from gel to liquid crystal phase, becoming leaky as they pass through the transition. Liposomes or isolated biological membranes dried with trehalose and rehydrated at room temperature, which is now above the dry T_m, do not experience a phase transition and suffer no damaging effects [4,12].

Bakers and brewers have known for years that dry yeast must be rehydrated with warm water if they are to remain viable. *S. cerevisiae* rehydrated at temperatures below 38–40°C suffer imbibitional damage, leak cytoplasmic contents into the rehydration media, and consequently suffer high mortality [13,14]. These results led Van Steveninck and Ledeboer [13] to propose that a gel to liquid crystal phase transition occurs when *S. cerevisiae* are rehydrated at low temperatures.

The presence of a phase transition in the upper

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range of yeasts' temperature tolerance may provide a strong clue as to the function of trehalose in dry yeast. It is reasonable that the T_m of membranes in dry yeast may be high, perhaps higher than yeast can physically tolerate, and that trehalose functions to lower the dry T_m into a physiologically tolerable range. In this study, the dry transition temperature of yeast membranes is determined and the effect of trehalose on the transition temperature is investigated.

2. Materials and methods

Yeast strains and culture conditions. Yeast strain 131 (*ras*2 mal) was a gift from Dr. A.D. Panek. Cultures were grown on standard 2% YED media (yeast extract 1.3%, glucose 2.0%, $(NH_4)_2SO_4$ 0.2%, and KH_2PO_4 0.2%) in an orbital shaker at 30°C and 160 rpm. Growth was followed by turbidity measurements at 560 nm. Cells in first log phase were collected after 15 h in culture, while stationary phase cells were removed after 48 h in culture (24 h after glucose exhaustion).

Extraction and measurement of trehalose. To extract trehalose, 10 mg dry weight of cells were collected by centrifugation ($500 \times g$ for 3 min), washed twice with 4°C distilled water, and incubated at 4°C in 2 ml of 0.5 M trichloroacetic acid for 20 min vortexing every 5 min. Trehalose in the acid extract was measured using an anthrone assay [15].

Dehydration. Cells were washed twice with 4°C distilled water, frozen in liquid nitrogen and placed on an Edwards Super Modulyo freeze-dryer for 24 h.

Measurement of leakage. Upon removal from the freeze-dryer, cells were rehydrated with 5 ml of distilled water at specific temperatures. Samples were allowed to return to room temperature (~ 25°C) before the conductivity was measured with a YSI model 35 conductivity meter and a model 3403 electrode.

Determination of survival. Survival was determined by rehydrating cells with distilled water at the desired temperature and then plating an aliquot on 2% YED plates. Plates were incubated for 48 h at 30°C before counting. Percent survival was calculated by comparing colony counts from experimental plates to control plates (no dehydration).

Isolation of plasma membrane. Starter cultures were prepared by loop inoculating 50 ml of liquid medium and grown as described above. 80 mg dry weight of exponentially growing cells from the starter cultures were used to inoculate 500 ml of 2% YPD. Cells were grown to a concentration of 4.5 mg dry weight/ml before harvesting (in early stationary phase). Plasma membranes were isolated following the method described in Araujo et al. [16].

Isolated plasma membrane preparations were separated into 1 mg protein aliquots, suspended in 1 ml water or 100 mM trehalose, frozen with liquid nitrogen and dried on a freeze-dryer for 24 h.

Determination of phase transition temperature. T_m of the dry cells and the dry membranes were determined using a Fourier Transform Infrared (FTIR) microscope connected to a Perkin-Elmer 1620 FTIR spectroscope. Dry samples were transferred under vacuum from the lyophilizer into a glove box flushed with dry nitrogen to a relative humidity of $9 \pm 3\%$. Samples were sandwiched between two CaF_2 windows and the edges were sealed with high-vacuum silicon grease. The sealed windows were removed from the glove box, loaded into a liquid nitrogen cooled temperature controller (Paige Instruments, Woodland, CA), and placed in the microscope. The sample chamber of the microscope was flushed with dry nitrogen for the length of each run. Sample temperature was increased in 2 C° increments, held constant for 1 min before scanning, and 30 scans were taken at each temperature. Scanning the entire temperature range took no longer than 2 h, and no hydration of the sample was detected over the course of the experiment.

T_m of the dry whole cells was determined by plotting the frequency of the PO_4 symmetric vibration against temperature, as described by [17]. The isolated membrane T_m was determined using the frequency of the CH_2 symmetric vibration [18].

Freeze-fracture. S. cerevisiae 14 membranes were prepared as describe above. Samples were frozen in liquid freon and fractured on a Balzers BAF 400D with platinum shadowing. Replicates were examined in a Philips 410 transmission electron microscope.

Lipid extraction. Lipid was extracted from an isolated membrane preparation by a chloroform/methanol (2:1, v/v) extraction [19]. After drying under N_2 the sample was resuspended in distilled water and the T_m determined as described above.

Measurement of ergosterol effects. Hydrated unilamellar DEPC (Avanti Polar Lipids, Birmingham, AL) liposomes were made by extrusion through polycarbonate filters (Poretics Co., Livermore, CA) and contained 0, 30 or 50% ergosterol (Sigma, St. Louis, MO) by weight. A Perkin-Elmer 1750 FTIR connected to a PC running Perkin-Elmers' IRDM software was used to determine the T_m of the liposomes. Except for loading the samples between the windows outside the drybox samples were treated as described above.

3. Results and discussion

3.1. Trehalose, rehydration temperature and survival

The importance of trehalose in *S. cerevisiae*'s ability to tolerate desiccation has been clearly demonstrated [8,20], and present results confirm and extend those

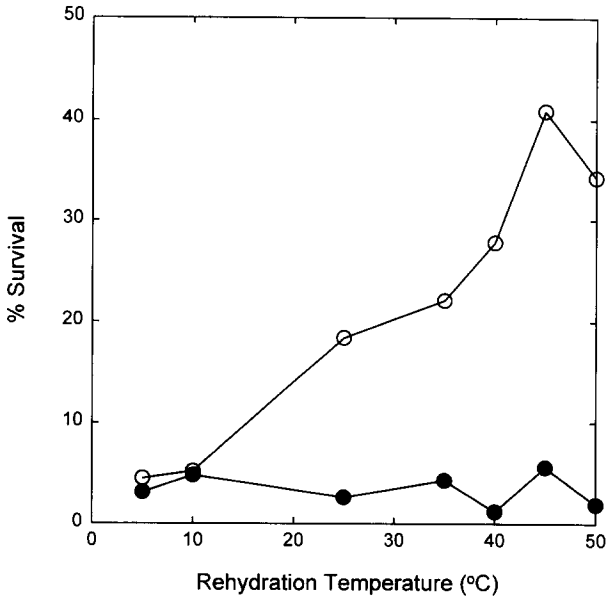

Fig. 1. Survival of yeast freeze-dried from stationary phase (○) or log phase (●) and rehydrated at various temperatures.

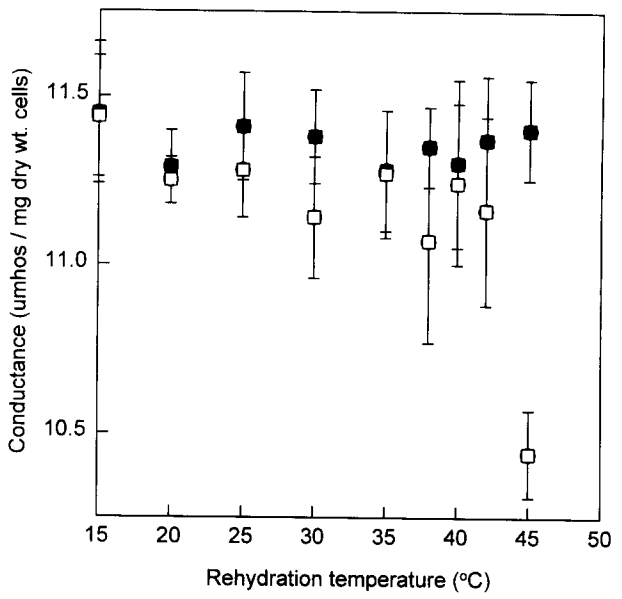

Fig. 2. Leakage from yeast freeze-dried from stationary phase (○) or log phase (●) and rehydrated at various temperatures as measured by conductance of the rehydration media. Vertical bars denote S.E. of the mean for four seperate determinations.

findings. *S. cerevisiae* 131 freeze-dried from stationary phase cultures (containing approximately 86 mg trehalose/g dry weight of cells) show a substantial increase in survival when they are rehydrated between 40–45°C while cells freeze-dried from the exponential phase of growth (with approximately 7 mg trehalose/g dry weight of cells) have low survival at all rehydration temperatures (Fig. 1). Leakage data show a similar pattern; stationary phase cells rehydrated below 40°C have higher leakage than cells rehydrated at higher temperatures and cells dried from log phase cultures have high leakage independent of rehydration temperature (Fig. 2).

3.2. Phase transitions and survival: intact cells

The high leakage and low survival of stationary phase cells rehydrated at lower temperatures have been attributed to a phase transition around 40°C [13], without direct evidence that this is so. The presence of a physiologically important phase transition offers an enticing explanation of the role of trehalose in dehydration tolerance in yeast. If trehalose serves to lower the membrane T_m in dry yeast to a physiologically tolerable range, around 40°C, thereby preventing the damaging effects of a phase transition in cells rehydrated above the T_m, it would explain both the increased survival and decreased leakage seen in stationary phase cells rehydrated above 40°C and the intolerance of log phase cells to rehydration at any temperature (Figs. 1 and 2).

To confirm the existence of a membrane transition around 40°C we attempted to use FTIR as previously done for artificial liposomes [4], pollen [21], and sperm [18]. Basically, the method consists of measuring the wavenumber of the CH_2 absorbance peak at various temperatures and then plotting the wavenumber against the temperature (Fig. 3). While this technique is very sensitive to changes in CH_2 vibrational frequency it cannot determine the source of the CH_2. If the primary source of CH_2 is phospholipid the transition

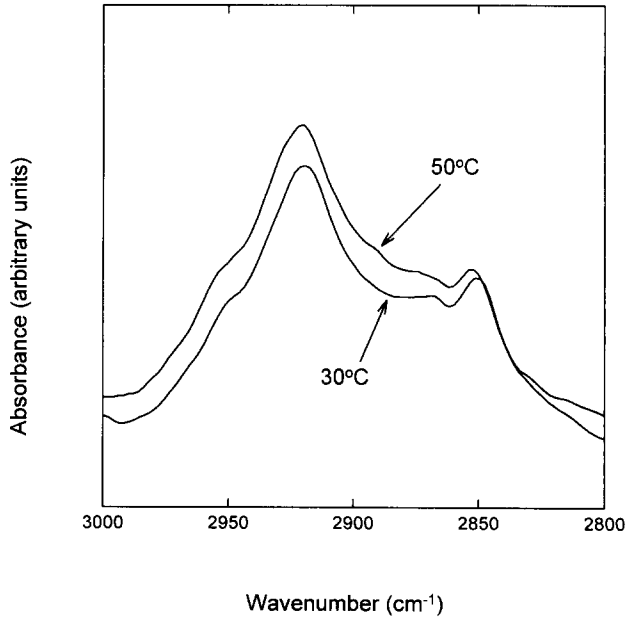

Fig. 3. Representative FTIR spectra of intact dry yeast showing the CH_2 peaks and the increase in vibrational frequency (wavenumber) seen with increasing temperature.

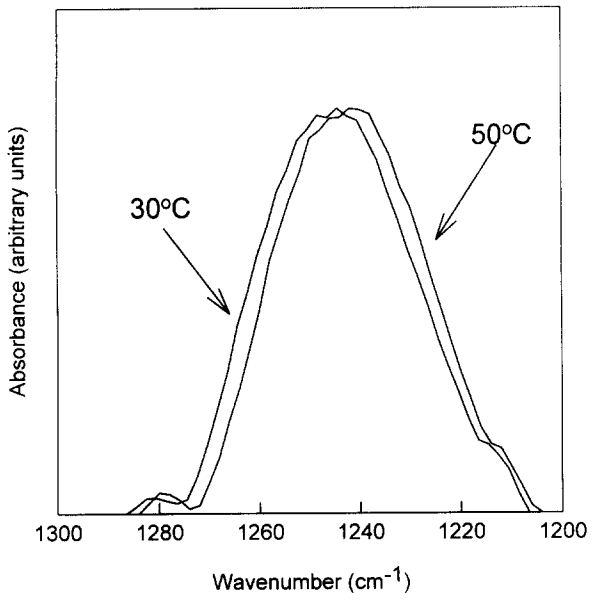

Fig. 4. Representative FTIR spectra of intact dry yeast showing the PO_2^- region and the decrease in vibrational frequency (wavenumber) seen with decreasing temperature.

determined will be that of the phospholipid membranes. However, if the majority of CH_2 is in the form of neutral lipid, such as triglycerols, the phospholipid transition is hidden under the stronger neutral lipid signal [21]. *S. cerevisiae* 131 contain large stores of neutral lipids in the form of lipid bodies, making it impossible to determine the T_m of the plasma membrane by observing the CH_2 symmetric stretch (data not shown). Previous work [17] has shown that in addition to the CH_2 symmetric stretch, the asymmetric phosphate stretch can be used to determine lipid phase changes. The phosphate absorbance peak from intact yeast is clear, undergoes a substantial shift when the membranes undergo a transition, and is not masked by the presence of neutral lipid in the cells (Fig. 4). Plotting the frequency of the phosphate vibration against temperature, it was possible to determine a T_m for intact dry yeast. Log phase cells, those without trehalose, have a T_m around 50°C, while stationary phase cells containing trehalose have a T_m around 30°C (Fig. 5). Although use of the phosphate vibration shows a transition, the T_m for stationary phase cells is lower than we would predict based on the survival and leakage data. The lower T_m could result from a change in headgroup packing prior to acyl chain melting, or it could be the result of inorganic phosphates in the cell, possibilities that are discussed in the next section.

3.3. Phase transitions in isolated membranes

To investigate the possible effect of inorganic phosphate on the measured phosphate transition, we determined the T_m of a plasma membrane preparation isolated from stationary phase cells. In addition to removing inorganic phosphate, the isolation procedure eliminates the problem of neutral lipid interference, making it possible to observe the CH_2 symmetric vibrations of the plasma membrane. Membranes dried without trehalose have a transition around 60°C, about 20 C° higher than membranes dried with trehalose (Fig. 5). Both of these values agree well with the survival and leakage data presented here (Figs. 1 and 2) and elsewhere [13,14] but are about 10 C° higher than the phosphate transition seen in intact cells. The difference between the phosphate and the CH_2 T_m values may indicate that phosphate headgroups undergo a change in packing or conformation prior to acyl chain melting. Alternatively, the higher temperature measured for the acyl chains may be a result of the membrane isolation [22]. However, when we determined T_m from the phosphate of the isolated membranes, we found that T_m is the same for intact cells and isolated membranes (Fig. 6). We suggest, based on this finding, that isolation of the membranes does not alter T_m and that the difference in apparent T_m as measured with the phosphate and CH_2 groups supports the idea that lipid headgroups undergo a conformational change before acyl chain melting.

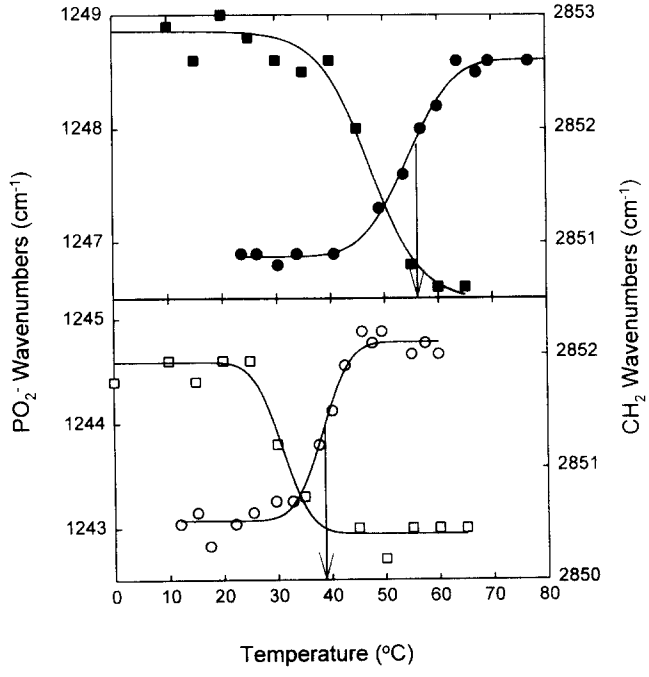

Fig. 5. Vibrational frequencies for the PO_2^- asymmetric stretch (□, ■) from intact yeast and the CH_2 symmetric stretch (○, ●) from isolated membranes dried with (□, ○) and without (■, ●) trehalose, as a function of temperature. Arrows indicate the transition temperature as determined from the CH_2 symmetric stretch of isolated membranes.

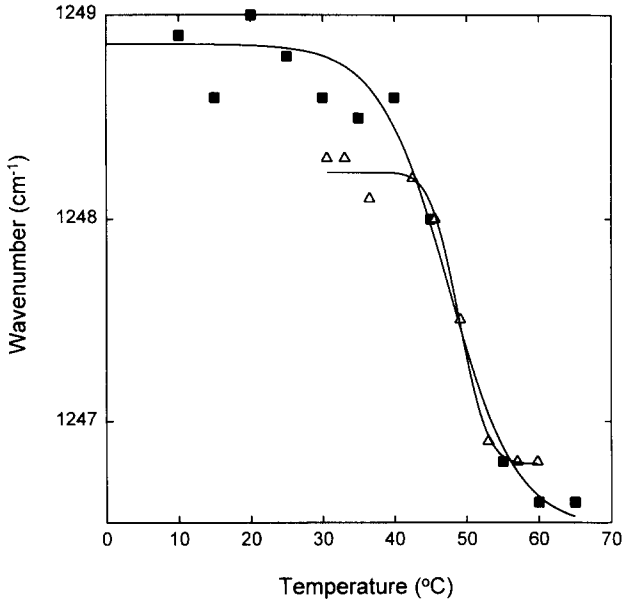

Fig. 6. Vibrational frequencies for the PO_2^- asymmetric stretch as a function of temperature in intact cells (■) dried from log phase and isolated membranes (△) dried without the addition of trehalose.

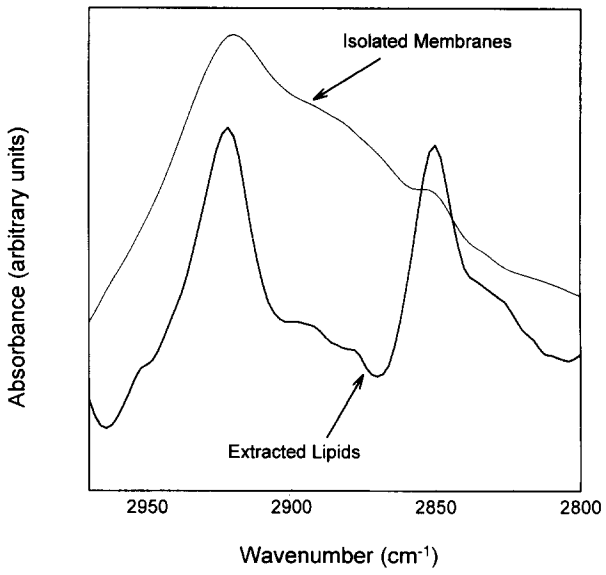

Fig. 7. Representative FTIR spectra at 35°C showing the CH_2 bands for isolated membranes and a lipid extract from the membranes. Although more easily seen in the extracted lipid, the CH_2 symmetric peak has the same vibrational frequency in both samples.

3.4. Phase transitions in extracted lipids

While it was possible to determine the CH_2 peaks in the isolated membranes we found that there was a much larger, unexplained peak in the CH_2 region of the spectra (Fig. 7). Freeze-fracture of membranes isolated from *S. cerevisiae* shows spikes extending from the membrane (Fig. 8), which we think are remnants of the cell wall not removed during the membrane isolation. By determining the T_m of lipid extracted from the isolated membranes via a chloroform/methanol (2:1) extraction we attempted to rule out the possibility that components of the cell wall remained in the membranes after the isolation procedure, influencing the T_m of the preparation, and to eliminate the unexplained peak seen in the isolated membrane spectra. Although

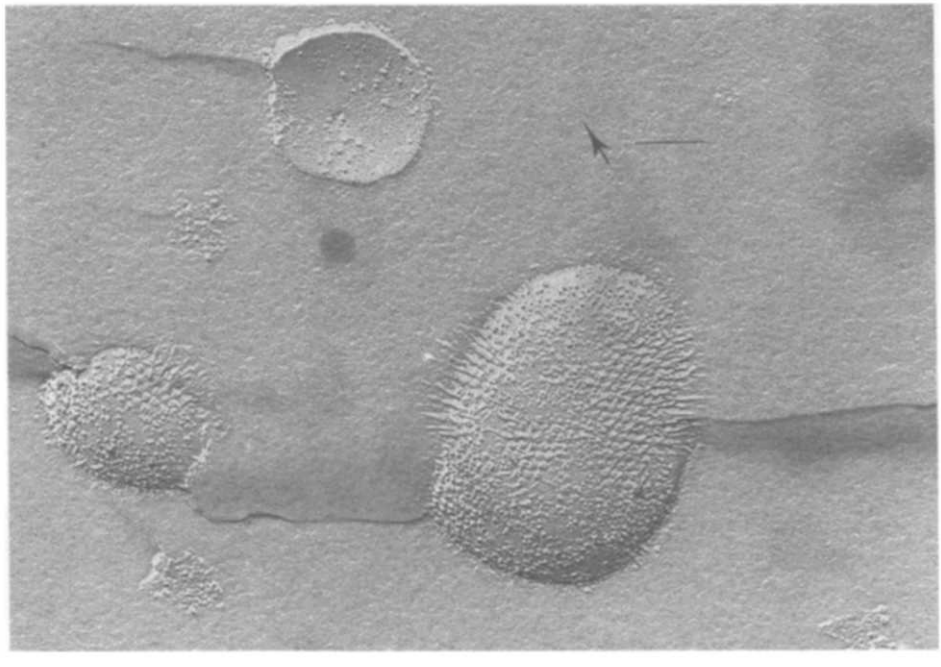

Fig. 8. Freeze-fracture image of isolated membrane from *S. cerevisiae* showing the spike-like structures present after the isolation procedure. These structures may account for the unusual appearance of the CH_2 bands in isolated membranes. The arrow indicates direction of shading and the bar represents 70 nm.

smaller, the T_m of the isolated lipid agrees well with that of the membrane preparation (Fig. 9), again confirming that the measured T_m is valid and not an artifact of the isolation procedure. Additionally, the large peak seen in spectra of isolated membranes was not present in the spectra of the extracted lipid (Fig. 7), in keeping with the suggestion that it is due to remnants of the cell wall.

3.5. Sterol effects on phase transitions

Extracted lipids have a T_m close to that of the isolated membranes, but the size of the transition (i.e., the change in vibrational frequency of the CH_2 groups) is substantially smaller than what is seen in the isolated membranes (Fig. 9). This magnitude of the frequency change seen in the isolated membranes is, in turn, smaller than that seen in pure phospholipids [4]. These effects could be due to the presence of sterols in the membranes. Increased sterol concentration makes the gel phase more fluid and the liquid crystal phase more rigid [23], resulting in a smaller change in frequency when the membrane undergoes a transition. In the case of extracted lipids, the difference in size and the slightly lower transition temperature compared with the isolated membranes may be explained by a change in the interaction between sterols and phospholipids following protein removal by the lipid extraction; in other words, isolation of the chloroform/methanol soluble components raises the sterol and phospholipid proportion of the membrane.

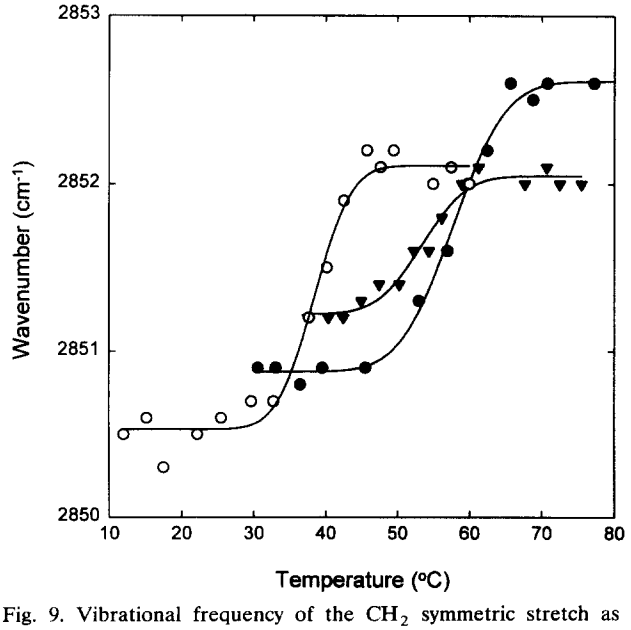

Fig. 9. Vibrational frequency of the CH_2 symmetric stretch as a function of temperature for isolated membranes dried with (○) and without (●) trehalose and extracted lipids (▼) dried without trehalose.

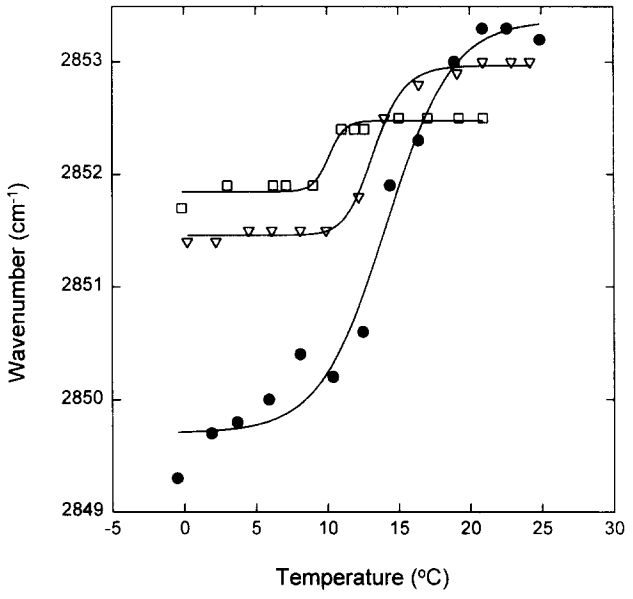

Fig. 10. Vibrational frequency of the CH_2 symmetric stretch as a function of temperature in DEPC unilamellar vesicles made with 0 (●), 30 (▽), or 50 (□) mol% ergosterol.

S. cerevisiae do not contain substantial amounts of cholesterol, but they do contain large amounts of ergosterol [24,25]. The effects of ergosterol on lipid phase transitions has not previously been investigated directly, but we expected, based on its structural similarities to cholesterol, that it would have similar effects on phospholipids. When phase transitions were measured in DEPC liposomes made with increasing amounts of ergosterol, the results shown in Fig. 10 were obtained. Clearly, as ergosterol concentration increases above 30% of the total lipid T_m is depressed and the magnitude of the frequency change is diminished, in keeping with the suggestion that ergosterol is responsible for these effects in the intact membranes (Fig. 5) and extracted lipids (Fig. 9).

3.6. Conclusion

In conclusion, this work shows that by lowering the dry T_m to a physiologically tolerable temperature trehalose is responsible for yeasts' ability to tolerate desiccation and subsequent rehydration and that a lipid phase transition is responsible for the high mortality seen in *S. cerevisiae* rehydrated below 40°C. Although not explicitly demonstrated in this study, it seems reasonable that trehalose may function in a similar role in other dehydration tolerant organisms.

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References

[1] De Virgilio, C., Simmen, U., Hottiger, T., Boller, T. and Wiemken, A. (1990) FEBS Lett. 273, 107–110.
[2] Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Science 223, 701–703.
[3] Panek, A.D. (1985) J. Biotechnol. 3, 121–130.
[4] Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Apell, L. (1985) Arch. Biochem. Biophys. 242, 240–247.
[5] Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Aurell Wistrom, C., Spargo, B.J. and Anchordoguy, T.J. (1988) Biochim. Biophys. Acta 947, 367–384.
[6] Wiemken, A. (1990) Antonie Van Leeuwenhoek 58, 209–217.
[7] Van Laere, A. (1989) FEMS Microbiol. Rev. 63, 201–210.
[8] Gadd, G.M., Chalmers, K. and Reed, R.H. (1987) FEMS Microbiol. Lett. 48, 249–254.
[9] Hottiger, T., Boller, T. and Wiemken, A. (1987) FEBS Lett. 220, 113–115.
[10] Mackenzie, K.F., Singh, K.K. and Brown, A.D. (1988) J. Gen. Microbiol. 134, 1661–1666.
[11] Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L. and Rudolph, A. (1986) Biochim. Biophys. Acta 861, 131–140.
[12] Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Wistrom, C. (1987) Biochem. J. 242, 1–10.
[13] Van Steveninck, J. and Ledeboer, A.M. (1974) Biochim. Biophys. Acta 352, 64–70.
[14] Pena, A., Uribe, S., Clemente, M. and Sanchez, N. (1992) Arch. Microbiol. 158, 75–80.
[15] Umbreit, W.M., Burris, R.H. and Stauffer, J.F. (1972) Manometric and Biochemical Techniques, 5th Edn., pp. 276–294, Burgress, Minneapolis.
[16] Araujo, P.S., Panek, A.C., Crowe, J.H., Crowe, L.M. and Panek, A.D. (1991) Biochem. Int. 24, 731–737.
[17] Crowe, L.M., Aurell Wistrom, C. and Crowe, J.H. (1992) Biophys. J. 61, A241.
[18] Crowe, J.H., Hoekstra, F.A., Crowe, L.M., Anchordoguy, T.J. and Drobnis, E. (1989) Cryobiology 26, 76–84.
[19] Kates, M. (1986) Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids, pp. 106–107, Elsevier, New York.
[20] Eleutherio, E.C.A., Araujo, P.S. and Panek, A.D. (1993) Biochim. Biophys. Acta 1156, 263–266.
[21] Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1989) Proc. Natl. Acad. Sci. USA 86, 520–523.
[22] Cameron, D.G., Martin, A. and Mantsch, H.H. (1983) Science 219, 180–182.
[23] Yeagle, P.L. (1991) Biochimie 73, 1303–1310.
[24] Rose, A.H. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.E., eds.), Vol. 2, pp. 308–327, Edward Arnold, London.
[25] Ratledge, C. and Evans, C.T. (1989) in The Yeast (Rose, A.H. and Harrison, J.S., eds.), Vol. 3, pp. 367–456, Academic Press, London.